OBSERVATIONS ON THE DEVELOPMENT OF HOOKWORM LARVAE

PART I

BY

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Neish (1913), in Jamaica, stated that ova and Ancylostome larvae were destroyed by normal saline, and on these grounds he explained the absence of hookworm infection on two estates in Portland, where the labourers' barracks were situated on the sea shore. Caldwell (1922), working in Honduras, suggests that the relatively low infection rate among the inhabitants of villages on the shore compared with that found in villages further inland is due to the lethal action of sea water on hookworm larvae. On the other hand, Nicoll (1917) found that even with a 6 per cent. solution of salt, several eggs were capable of hatching after as long as six or seven days in the salt. Nicoll's results suggest that it is something besides the mere presence of salt which prevents the development of hookworm eggs and larvae, and the following series of experiments were undertaken with a view to finding out, if possible, what was the real reason underlying the observations of Neish and Caldwell.

As Maplestone (1924) has shown, *Necator americanus* is much commoner than *Ancylostoma duodenale* in Freetown, and the following results should be taken as applying to the former species. Artificial cultures were used throughout, for although the soil in and around Freetown must be heavily infected with the larvae of *N. americanus*, the results would be unreliable if naturally infected soil were used, because the numerous cats and dogs present being heavily infected with *A. caninum* and *A. ceylanicum*, these larvae would be found in large numbers as well, and Stekhoven and Stekhoven-Meyer (1924) have shown that the larvae of these two species are indistinguishable from those of the worms infecting human beings. In this connection, the writer is not aware that *A. caninum* and *A. ceylanicum* are present in the Islands of Trinidad and Porto Rico, but it is a remarkable fact that in the numerous papers of the series ' Investigations on the Control of Hookworm Disease,' recently published by Cort and many other American workers in these places, the possibility of confusing the larvae of *N. americanus* with those of the above species is not referred to.

TECHNIQUE

All the work was done in the laboratory at room temperature. The cultures were placed on a bench beneath large windows and were covered with glass bell jars ; they thus received plenty of light but were never in direct sunlight.

Except in a few special cases cultures were always examined on the seventh day of growth, so that comparison between them would be as accurate as possible.

The materials used in the cultures were powdered wood charcoal, laterite, which is the natural soil in Freetown, and sand taken from the sea shore below high-tide level. The laterite and sand were heated in enamel saucepans to between 60 and 70 degrees Centigrade for about twenty minutes. This temperature did not dry or char the material and was always found sufficient to destroy all nematodes, whether larvae or adults, that were in the soil. They were then passed through a sieve with a mesh of 4 mm., to remove the larger stones. The materials were stored in tins, and in a few days the laterite became perfectly dry, whereas the sand remained moist for months. The sand was found to be slightly alkaline (pH 7.6) and the laterite was neutral in reaction. The relative weights of charcoal, laterite and sand were 2, 3, and 9, respectively per volume, therefore quantities for making cultures were measured by volume and not by weight, so that all cultures were of equal size and the mixture of faeces and culture medium were in the same proportions on all occasions. The amount of faeces used in a culture was always 3 grams. It was soon found that charcoal mixed with laterite was no better than plain laterite as a culture medium, so that the charcoal was discontinued.

Wire gauze with a mesh of 1.158 mm. was made into small baskets measuring 4 cm. square and 2 cm. in depth, thus having a capacity of 32 c.c. Small cultures, consisting as they did of 25 c.c. of earth and 3 grams of faeces, easily fitted into these baskets. For isolation of larvae from the cultures the baskets, as a whole, were placed in the isolation apparatus. The advantages of this method were that handling of cultures was simple and rapid, and there was no danger of loss of any of the culture by transferring it to a fresh sieve ; the wire gauze also allowed water to drain away freely, so the cultures were never standing in excess water, being unduly waterlogged in consequence. For some cultures on a larger scale that it was found advisable to make, wooden boxes in the shape of cubes, 10 cm. × 10 cm., were made.

For isolation of larvae from cultures, funnels of suitable size for the culture concerned were fitted with a short piece of rubber tubing closed by a spring clip, in every way similar to the apparatus used by Cort *et al.* (1922). The funnels were lined with a double layer of plain white muslin as it was found that a single layer let too much soil through. The method of dealing with the small cultures has already been described ; for the large cultures wire gauze sieves of suitable size were made, using gauze of the same mesh as that from which the small baskets were made. The cultures in the latter case had to be transferred from the boxes to the sieves. After being placed in a suitably sized funnel and the spring clip being applied to the rubber tubing, water at a temperature of 50° C. was poured in until the soil was partly submerged. By starting with water at 50° C. it was reduced to about 45° C. by the time it had come in contact with the culture ; this temperature has been shown by Cort *et al.* (1922) to be the optimum for the isolation of larvae, and a few preliminary experiments confirmed this. The water was poured carefully down the side of the funnel, avoiding contact with the culture, until it rose sufficiently high to reach the soil from below. A series of cultures was always put up for isolation in the afternoon and examined on the following morning, that is, fifteen to eighteen hours afterwards.

Many cultures produced thousands of larvae, which rendered counting all of them impossible, so the following method was adopted. Centrifuge tubes holding about 15 c.c. were marked at 10 c.c. and 5 c.c. The clip at the end of the rubber tube was slowly released, allowing exactly 10 c.c. of water from the culture to flow into the tube. This amount of water was found to contain all the larvae that had come out of the culture. The tube was then centrifuged to throw all the larvae to the bottom and then 5 c.c. of water were drawn off, leaving all the larvae from a given culture in 5 c.c. of water. A Wassermann pipette, cut short to facilitate handling, was fitted with a rubber teat, and this was used for collecting samples for counting. An even suspension of the larvae was first made by drawing up and forcibly expelling portions of the contents of the tube; using the pipette for the purpose, it could easily be determined by the naked eye when this had taken place. The pipette was now filled and 0·1 c.c. allowed to run on to a slide. This operation was repeated five times and five slides from each culture were prepared; but after the taking of each 0·1 c.c. sample, care was taken to mix the suspension of larvae thoroughly, because they were found to settle to the bottom very rapidly. The number of larvae in each of the five drops was counted, the five totals added, and this figure being multiplied by ten gave the number of larvae in the original 5 c.c. of water, that is, the number of larvae isolated from the culture. A cover slip was found unnecessary as the larvae could be counted easily with a low power (Zeiss objective A, and eyepiece 4). In counting, a mechanical stage was used and the whole drop of water passed under review; to avoid counting larvae more than once or missing them altogether, those larvae that only partly showed in the lower edge of the field were counted and those that only partly showed in the upper edge of the field were omitted. A drop of Lugol's solution was added before counting because it rendered the larvae motionless at once; this was found necessary for, with a large number of larvae wriggling actively about, it is not possible to count them accurately, and a further possible source of error is that some larvae may pass out of the field either before or after being counted, and the same larvae might thus be encountered in another part of the drop. Another advantage of the use of Lugol's solution is that it enabled the distinction between hookworm and Strongyloides larvae to be made rapidly and accurately; this is a simple matter in the ordinary way, but when there are large numbers of larvae wriggling about and some of the hookworm larvae have lost their sheaths, it is not so easy to distinguish between them.

In the following experiments it will be noted that the numbers of larvae isolated in different series of cultures are very different; this is because faeces from many individuals were used during the course of the work.

For the sake of brevity all the experiments given below are expressed as a single figure which, however, represents the average of a large number of individual experiments.

SECTION I. The Effect of Sea Water and Sea Sand on the Development of Hookworm Eggs and Larvae.

EXPERIMENT 1.

Portions of faeces 3 grams in weight were mixed with laterite and charcoal, half of the cultures were kept moist with sea water, and half with tap water as controls.

Cultures moistened with sea water produced 313 larvae per gram of faeces, and the controls moistened with tap water produced 328 larvae per gram of faeces.

This clearly shows that sea water alone has no effect on the development of hookworm eggs and larvae.

EXPERIMENT 2.

The effect of sea sand was next tried, being controlled by cultures of faeces in charcoal and laterite mixture, and all cultures were moistened with tap water. Sand and laterite prepared in the manner described were used throughout. In a series of over twenty different experiments on these lines, using faeces from several individuals, the number of larvae per gram of faeces recovered from sand cultures varied from nil to about 20 per cent. of the numbers recovered from the charcoal-laterite controls.

Next, a series of seven cultures in sand and a similar number of controls in charcoal-laterite mixture were put up. One each of these cultures was examined every day from the first to the seventh day of growth, and it was found that on the first and second days the sand cultures and the controls produced an approximately equal number of larvae; but from the third day onwards the numbers in the sand cultures gradually diminished, whilst the controls remained unaltered. This clearly indicated that, whatever the action of the sand might be, it was exerted on the young larvae and not on the eggs before hatching.

A portion of previously heated sea sand was washed by allowing tap water to percolate through it for twenty-four hours. It was then dried and used as a culture medium, controlled by cultures in charcoal-laterite and unwashed sea sand. In addition to mixing with sand alone, various modifications such as mixing faeces with sand-charcoal-laterite in various proportions or covering cultures with a layer of one or other of the media, were also tried. Three grams of faeces were used in all cases, the cultures were grown for seven days, and the results are expressed in terms of the number of larvae isolated from one gram of faeces. In the following series of experiments unwashed sea sand is called ‘ sea sand,’ washed sea sand is called ‘ washed sand,’ and charcoal-laterite mixture is called ‘ earth.’

Experiment 2a.

Faeces-earth-washed sand in equal parts produced	400 larvae.
Faeces-earth-sea sand in equal parts produced	1,440 larvae.
Faeces-earth, covered with an equal volume of washed sand produced	530 larvae.
Faeces-earth, covered with an equal volume of sea sand produced	90 larvae.

Experiment 2b.

Faeces-washed sand produced	143 larvae.
Faeces-sea sand produced	27 larvae.

Experiment 2c.

Faeces-earth not covered produced	295 larvae.
Faeces-earth covered with washed sand produced	108 larvae.
Faeces-earth covered with sea sand produced	79 larvae.
Faeces-washed sand not covered produced	102 larvae.
Faeces-sea sand not covered produced	10 larvae.

Experiment 2d.

Faeces-earth not covered produced	920 larvae.
Faeces-earth covered with an equal volume of same produced	957 larvae.
Faeces-earth covered with an equal volume of washed sand produced	727 larvae.
Faeces-earth covered with an equal volume of sea sand produced	100 larvae.

Experiment 2e.

Faeces-earth produced	860 larvae.
Faeces-earth (1 part)-sea sand (1 part) produced	27 larvae.
Faeces-earth (1 part)-sea sand (2 parts) produced	37 larvae.
Faeces-sea sand produced	0 larvae.

The above series of experiments indicates that sea sand has a lethal effect on hookworm larvae when used under the conditions of these experiments, and they show that unwashed sand is more powerful than washed sand. It was now decided to test the same materials, using the same amount of faeces but much larger amounts of earth and sand, and for this purpose wooden boxes 10 cm. square by 10 cm. in depth were employed in place of the small wire gauze baskets. The boxes were filled with laterite to within one inch of the top, the faeces was placed on the surface and covered with a thin layer of the material to be tested. From now on charcoal-laterite mixtures were replaced by plain laterite as it was found that the latter was a slightly more favourable culture medium.

EXPERIMENT 2f.

Faeces-earth covered with laterite half-inch and moistened daily produced	446 larvae.
Faeces-earth covered with sea sand half-inch and not moistened produced	483 larvae.
Faeces-earth covered with sea sand half-inch and moistened daily produced	518 larvae.

This series of experiments indicates that sea sand seems to be effective only in small cultures and not in large ones ; this is difficult to understand as, with a much greater proportion of sand to faeces, it might be expected that the effect would be more marked in large cultures than in small ones. As a check on this contradictory result a fresh series of small cultures was made in the wire baskets.

EXPERIMENT 2g.

Faeces-earth covered with earth produced	382 larvae.
Faeces-earth covered with sea sand produced	346 larvae.

This apparently showed that the sand had lost its former effect, and as the sand used up to this time had been stored in the laboratory for some months it was thought this might be the reason. Accordingly a fresh lot of sand was procured and after being heated in the same way as the first lot it was tried under identical conditions.

EXPERIMENT 2h.

Faeces-earth covered with earth produced	5,612 larvae.
Faeces-earth covered with old sand produced	5,042 larvae.
Faeces-earth covered with new sand produced	3,050 larvae.

This showed that the new sand was more effective than the old sand, but it was not nearly so powerful as the old was when first collected.

EXPERIMENT 2j.

Testing the new sand in larger quantities, viz., in boxes.

Faeces-earth covered with earth produced	1,327 larvae.
Faeces-earth covered with new sand produced	933 larvae.

Reduction of the results of the two experiments 2h and 2j, to the same proportion, gives 1.5 : 1.47, which shows that the effect of the new sand is practically the same in small and large cultures. But in less than a week it was found that the new sand was as ineffective as the old.

All the experiments hitherto recorded have been subjected to the same conditions of moisture except where the contrary is stated, and the cultures in any given series have always been wetted with equal amounts of water and at the same time.

The foregoing experiments were carried out from August to the beginning of November, at which time the sand was found to have become ineffective. The reason for the loss of power in the sand was puzzling until it was realised that the change in its efficacy coincided with the change from the wet to the dry season. During the rains in Sierra Leone, humidity is high and evaporation is, consequently, slight. It had been noted that sand cultures were always more sodden with water than corresponding earth cultures, even when equal amounts of water were used for moistening them, suggesting that earth is able to absorb more water than sand. The idea, therefore, suggested itself that the suspected lethal effect was not in the sand *per sé* but was due to the inability of the sand to absorb water as readily as earth, with consequent excess of water in the former type of cultures. This excess was more marked in the wet than in the dry seasons for, during the latter, the greater evaporation would allow the sand cultures to approximate more nearly to the earth cultures with respect to the amount of free water present. This hypothesis was supported by the observation that, in boxes which contained a large quantity of earth below the cultures, the sand was not effective at all, and this could be explained as follows :—The large quantity of absorbent earth beneath the sand drained excess of water out of the latter, thus allowing the sand to become a suitable culture medium.

The next series of experiments was devised with the object of testing the accuracy of the above theory. Two cultures were put up in sand and two in laterite, one of each being used as controls. In this case the controls had only enough water added to keep them just moist, the sand control requiring much less water than the laterite control. The other cultures were freely moistened daily so that they were always in a state of saturation. This experiment was repeated six times and the results are expressed as an average of the whole.

Experiment 3a.

Faeces-laterite kept just moist produced	303 larvae.
Faeces-sand kept just moist produced	300 larvae.
Faeces-laterite freely moistened produced	117 larvae.
Faeces-sand freely moistened produced	120 larvae.

This clearly shows that sea sand and earth, when under equal conditions of moisture, are of the same value as culture media, and as a corollary of this it is evident that, when moistened with equal quantities of water, sand appears a worse medium than laterite because of its inability to absorb water as readily as the latter. Further confirmation of this was obtained by the following series of experiments. They were devised with the object of obtaining as nearly as possible the condition of faeces lying on the ground in the water-logged soil at the edge of a stream or pond.

Experiment 3b.

Small glass jars about 15 cm. in height and 3 cm. in diameter were filled with laterite to within about 4 cm. of the top. Portions of faeces, 3 grams in weight, were mixed with 25 c.c. of laterite and placed on top of the laterite in the jars. The surface of the culture was not flat but was sloped upwards to one side of the jar, so as to reach nearly to the top. Water was now poured into the jar until it just reached the junction of the original soil and the culture, and it was kept at this level continuously by making good every day any loss from evaporation. The effect of this was that the cultures were always completely saturated with water but were not immersed in it. A similar set of jars was prepared, using sea sand instead of laterite, and sea water instead of tap water. For examination the cultures were carefully removed from the surface of the original soil in the jars and were placed in wire baskets for isolation in the usual way. The cultures were then drained off, allowed to remain in the baskets for another week, being kept just moist meanwhile, and they were then tested by isolation a second time. Two series of cultures were prepared, one being kept in the jars for a week and the other for two weeks before isolation.

Series 1 (kept for seven days).

Laterite culture produced...	50 larvae.	1st isolation.
Sand culture produced	7 larvae.	
Laterite culture produced...	67 larvae.	2nd isolation.
Sand culture produced	197 larvae.	

Series 2 (kept for fourteen days).

Laterite culture produced...	230 larvae.	1st isolation.
Sand culture produced	20 larvae.	
Laterite culture produced...	0 larvae.	2nd isolation.
Sand culture produced	223 larvae.	

Control cultures in gauze baskets produced 820 larvae, and as the residual soil in the jars did not contain any larvae it is clear that saturation of soil containing faeces is sufficient to reduce greatly the number of larvae that will develop from it even if brought into more favourable surroundings later on. Payne (1922) has noted the fact that the death rate of hookworm larvae is high in water-logged soil, and the foregoing experiments seem to be a clear confirmation of this.

CONCLUSION

The evidence of the foregoing experiments tends to show that sea water and sea sand *per se* have no effect in preventing the development of hookworm eggs and larvae.

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